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Phosphoinositide metabolism in hereditary ovalocytic red blood cell membranes

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Abstract

Metabolic depletion of hereditary ovalocytes leads, similar to normal red cells, to decreased intracellular concentrations of ATP and GSH as well as degradation of the phosphoinositides to phosphatidylinositol and diacylglycerol. In contrast to normal red cells, however, loss of ATP does not induce any gross shape transformations; even after extensive depletion the ovalocytes retain their initial elongated stomatocytic character. The mechanical properties of hereditary ovalocytes are associated with a deletion of nine amino acid residues in band 3. Since the deletion appears to increase the stiffness of a normally flexible region of band 3, connecting the N-terminal cytoplasmic domain with the membrane spanning domain, our results indicate that shape changes require a flexible attachment of the cytoskeleton to the membrane-spanning band 3. The results also imply that metabolism of phosphoinositide cannot be the only determinant of cell shape, as suggested by the bilayer-couple hypothesis, but also other factors are involved in metabolically induced shape transformations.

Keywords: Erythrocyte; Ovalocytosis; Shape change; Membrane; Phosphoinositide metabolism; (Human)

1. Introduction

When the red blood cell travels around the vascular system the cell has to withstand not only considerably mechanical stress caused by turbulence in the heart but also the shearing forces in the thin capillaries. The remarkable ability to passively change shape without disruption allows the red cell to survive the ferocious environment. This essential quality requires the membrane to be deformable, elastic and stable. The membrane cytoskeleton, which lines the cytoplasmic surface of the red cell membrane, and the membrane lipid bilayer are believed to be responsible for the mechanical properties and elasticity of the red cell [1–3]. Mutations, deletions or loss of cytoskeletal proteins result in impaired protein–protein associations and lead to anomalies in rigidity, cell shape and reduced stability of the membrane [4,5].

South East Asian ovalocytes are oval-shaped erythrocytes that are more rigid and less deformable than ordinary red cells [6–9]. The anomaly of hereditary ovalocytes is associated with the deletion of nine amino acids, from residue 400 to 408, at the boundary between the N-terminal cytoplasmic and membrane domains, in about 40% of the band 3 molecules present in ovalocytic membranes [10–12]. It is likely that the homozygous state is lethal as no homozygous individual is known. Since red cells of heterozygotes

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are less vulnerable to malaria invasion in vitro, it has been suggested that hereditary ovalocytosis confer some protection against malaria [13,14]. However, more recent data indicate that fresh ovalocytes are invaded by the parasite nearly as well as normal red cells [15]. The deletion mutation is linked to a point mutation at amino acid 56 (Lys to Glu), which also occurs in a common asymptomatic band 3 variant known as band 3 Memphis [16–18].

When a normal red blood cell is depleted of its ATP, a series of shape changes occur. At the early stages, bumps appear on the cell membrane. Later these bumps change into spicules and the discoid character is lost in favour of a spherical form. If the process is allowed to continue, the spicules are elongated and, in due course, lost as microvesicles with the formation of a smooth spherical cell. The shape changes can be reverted up to the point of membrane loss by allowing the cell to synthesize ATP [19–21]. Metabolic depletion under many different conditions have shown that the shape transformation parallels the dephosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP_2), phosphatidylinositol 4-monophosphate (PIP) and phosphatidic acid (PA) [22,23]. Since these phospholipids are localized mainly to the inner leaflet of the membrane [24], dephosphorylation of the phosphoinositides would reduce the area of that leaflet, forcing the outer leaflet to produce the protrusions characteristic of echinocytosis. This behaviour is in accordance with the bilayer-couple hypothesis that explains the cell shape as a function of the relative areas of the inner and outer leaflets of the membrane [25,26].

Although the shape change behaviour of hereditary ovalocytes has not been studied previously, it seemed likely that metabolic depletion of these abnormally rigid cells might lead to different shape transformations. Assuming that the bilayer-couple hypothesis is correct, it follows that the dephosphorylation of phosphoinositides in ovalocytes during depleting conditions should also be impaired.

In this study we have investigated the effect of metabolic depletion on the shape and phosphoinositide metabolism of hereditary ovalocytes. We found that shape changes occurred in hereditary ovalocytes, though to a much lesser extent than in normal cells, and that the metabolism of the phosphoinositides was very similar to that observed in normal red cells.

2. Materials and methods

Fresh human blood was kindly provided by the blood bank at the University Hospital, Umeå. The clinical and laboratory details of the donor of the ovalocytes and his identical twin brother have been reported previously [27]. Briefly, the donor presented with clinical symptoms associated with a well-compensated, mild hemolysis. The characteristic red cell morphology, protein and DNA abnormalities of band 3 in South East Asian ovalocytosis were detected in both cases. A similar clinical history was recounted for paternal relatives. Normal red cells were drawn at the same time and, exactly as the ovalocytes, transported from England to Sweden. Due to the transportation 4 days elapsed before the start of the experiments. Therefore these normal red cells were termed 'old' control cells. Essentially fatty acid-free bovine serum albumin (BSA) and glutaraldehyde were from Sigma. Penicillin G-streptomycin mixture was from Gibco. Carrier-free [^{32}P]Orthophosphate was purchased from Amersham. Silica gel 60 thin-layer chromatography plates (20 cm × 20 cm) were from Merck. All other chemicals were of at least reagent grade.

Red cells were washed five times with ice-cold buffer H (130 mM NaCl, 3.7 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 25 mM Hepes, pH 7.5) and twice with ice-cold complete buffer H (buffer H containing 100 units ml^{-1} penicillin G, 100 units ml^{-1} streptomycin and 1 mg ml^{-1} BSA). Plasma and buffy coat were removed by aspiration after each centrifugation.

Red cells (10% haematocrit) were labelled with [^{32}P]orthophosphate (0.74 MBq ml^{-1}) by incubation for 25 h at 37°C in complete buffer H supplemented with 10 mM glucose and 1 mM adenosine. After incubation, cells were washed four times with buffer H and twice with complete buffer H.

Metabolic depletion was initiated by resuspending ^{32}P-labelled cells to 10% haematocrit in complete buffer H.

Cells were fixed by mixing 30 μl of the red cell suspension in 0.2 ml 1% glutaraldehyde in 0.1 M potassium phosphate buffer (pH 7.5) for 60 min at room temperature. The morphological index was determined by phase contrast microscopy, counting at least 400 cells of each sample. Discocytes and echinocytes were assigned a morphology score of 0

and 1–4, respectively [28] and the average score of each sample was taken as the morphological index.

For scanning electron microscopy, fixed cells were washed in phosphate-buffered isotonic saline and 50 μl of each sample was placed onto glass coverslips pre-coated with poly-L-lysine [29]. The coverslips were gently rinsed with isotonic saline, dehydrated in increased concentrations of ethanol and dried from CO_2 using critical point technique [30]. Coverslips were mounted on aluminium stubs and coated with gold using a combined system of sputter-coating (Edwards S150A Sputter Coating Unit, Edwards High Vacuum) and a modified vacuum coating system equipped with an automatic rotation and tilting device (Edwards E14 Vacuum Coating Unit). All specimens were analyzed in a Leo S-360 iXP scanning electron microscope (Leo Electron Microscopy Ltd.) fitted with a LaB6 emitter (Kimbal Physics Inc.) running at standard settings. Micrographs were taken from all samples at an original magnification of 2500 and 5000 times.

ATP and GSH were determined as described previously [23]. The concentration of haemoglobin in lysates was determined from measurements of absorbance at 539 nm using a molar absorbtivity of 13.4×10^3 M^{-1} cm^{-1} per haem [31].

Lipids were extracted from frozen samples of cell suspension using acidic methanol/chloroform and separated by thin-layer chromatography as described before [23]. Radiolabelled phospholipids were detected by autoradiography and quantified by liquid scintillation counting of scraped spots. Lipid phosphorus was determined spectroscopically [32].

3. Results

Incubation of ovalocytes, in the absence of nutrients such as glucose, caused depletion of both ATP and GSH, as shown in Fig. 1. Both hereditary ovalocytes and control cells treated in exactly the same way (i.e., drawn at the same time and transported from England to Sweden) had lower initial levels of ATP (0.56 and 1.05 μmole/g haemoglobin, respectively) and GSH (3.7 and 4.6 μmole/g haemoglobin, respectively) even after repletion with glucose and adenosine compared to fresh control cells (4.1 μmole

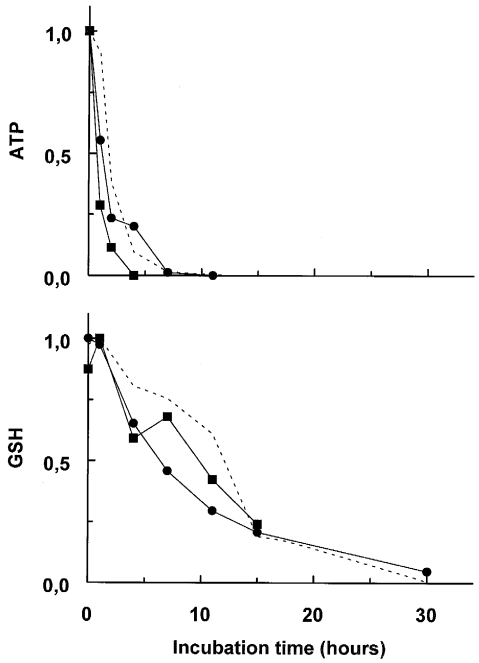

Fig. 1. ATP- and GSH-levels during metabolic depletion of human red cells. Washed red cells were suspended at 10% haematocrit in complete buffer H and incubated at 37°C. At the indicated times samples were withdrawn and the concentration of ATP (top) and GSH (bottom) were determined as described in Section 2. 100% ATP represent 0.56 μmole/g haemoglobin in ovalocytic red cells (■) and 1.05 μmole/g haemoglobin in 'old' control cells (●). The initial concentrations of GSH were 3.7 and 4.6 μmole/g haemoglobin in ovalocytes (■) and control cells (●). The dotted lines represent the time course of changes in ATP (4.1 μmole/g haemoglobin) and GSH (7 μmole/g haemoglobin) of fresh control cells.

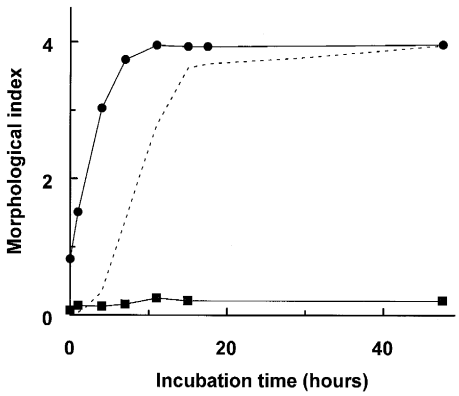

Fig. 2. Time course of shape changes during metabolic depletion. Samples were withdrawn at the indicated times and fixed by glutaraldehyde. The extent of crenation of ovalocytes (■) and 'old' control red cells (●) was determined by phase contrast microscopy. The shape changes of fresh red cells (dotted line) were also determined.

ATP/g haemoglobin and 7 μmole GSH/g haemoglobin). The lower initial level of ATP has been observed previously [15] and can probably be attributed to an enhanced energy consumption due to a membrane leakage of cations in ovalocytic cells. Even though the initial concentrations of ATP and

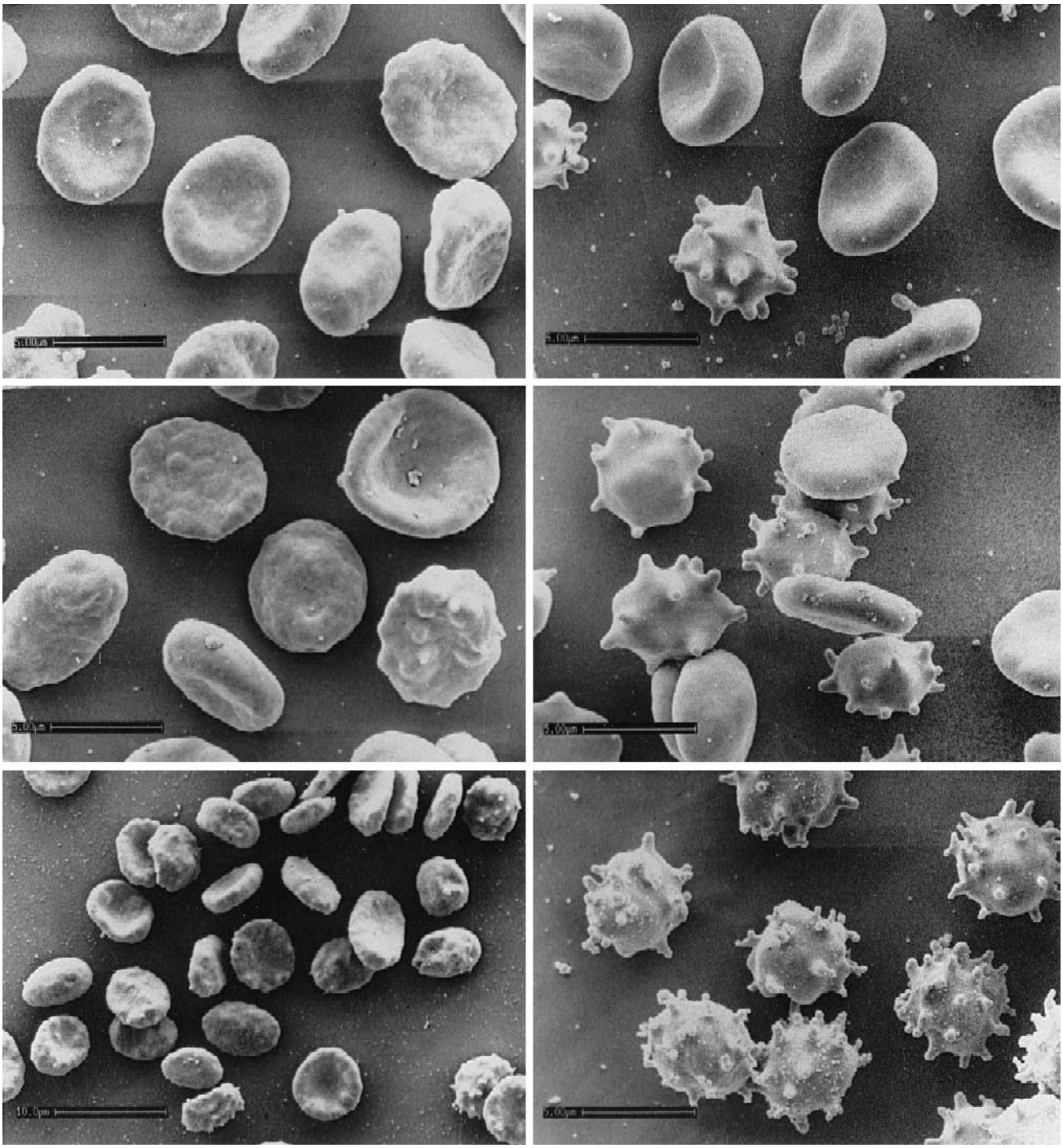

Fig. 3. Scanning electron microscopy of red cells. Samples of hereditary ovalocytes (left column) or 'old' control red cells (right column) were withdrawn after 0 h (top row), 1 h (middle row) and 13 and 7 h (ovalocytes and control cells, respectively; bottom row) metabolic depletion and prepared for electron microscopy. Scale: Bar represents 5 μm in top and middle row and 10 μm in bottom row.

GSH were lower in the ovalocytes, the relative changes in ATP and GSH were very similar to those observed both in 'old' (i.e., transported) and fresh control red cells. Whether the slightly faster decline in the relative ATP level of ovalocytes was a genuine effect or due to increased membrane leakage is unknown at present.

Independent of the pre-treatment, control cells lost their discoid shape upon incubation under ATP-depleting condition. The normal biconcave shape was gradually lost in favour of various states of spiculated cell shapes. Eventually, the typical spikes of echinocytosis were shed, giving rise to a smooth spherocyte. The 'old' control cells went through a quicker echinocytosis than fresh control cells, lacking the usual lag phase initially (Fig. 2). These cells reached a morphological index of 2 after about 4–5 h, whereas fresh cells required about 10 h metabolic depletion to reach the same stage of shape transformation.

In striking contrast to the control cells, by phase contrast microscopy it was not possible to observe any noticeable shape transformation of ovalocytic red cells upon ATP-depletion; even after extensive metabolic depletion the morphological index only reached 0.2 (Fig. 2). However, when the same cells were observed by scanning electron microscopy, it became evident that metabolic depletion did indeed cause substantial shape transformation, also of ovalocytes, although the extent of transformation was much less pronounced. Ovalocytes did not go through the typical stages of echinocytosis, including loss of biconcave shape and development of protrusion and spikes on the membrane surface. Instead these cells developed an undulated pattern of bumps or bulges on the membrane surface, as shown by Fig. 3. Even after extensive depletion (i.e., in the absence of ATP) the ovalocytes retained the stomatocytic character.

The lack of any major shape changes in ovalocytes seemed to indicate, when interpreted in accordance with the bilayer-couple hypothesis, that the relative areas of the outer and inner leaflet of the membrane did not change upon metabolic depletion. Since degradation of the phosphoinositides are believed to be the major cause of area change of the two leaflets during depletion [22,23], our results imply that the phosphoinositide metabolism of ovalocytic red cells differs from normal red cells. To investigate this possibility, ovalocytes were labelled by preincubation for 24 h with nutrients and radioactive orthophosphate, a treatment that incorporates the label into PIP_2, PIP and PA as well as several proteins [22,23,33,34]. As expected, among the phospholipids only PIP_2, PIP and PA were labelled.

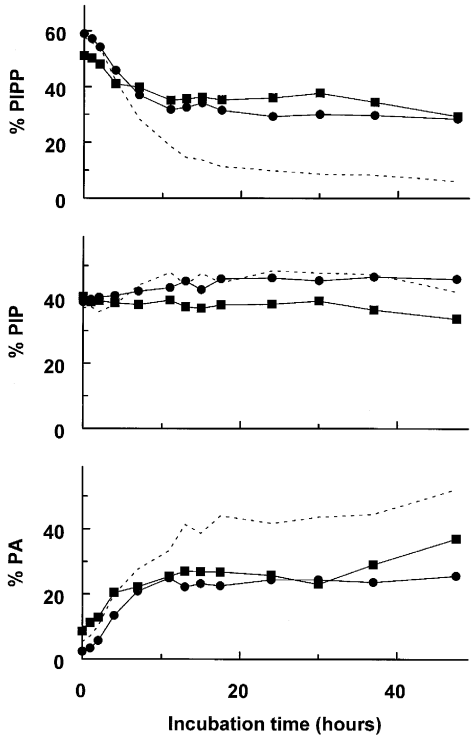

Fig. 4. Metabolism of the phosphoinositides during metabolic depletion. Labelled red cells were incubated at 37°C in the absence of nutrients to induce depletion. Samples were withdrawn and analyzed as described in Section 2, and the fractions of PIP_2, PIP and PA from ovalocytes (■), 'old' control cells (●), and fresh control cells (dotted line) were determined.

In spite of the atypical shape changes, the phosphoinositide turn-over of the ovalocytes did not differ significantly from that of the 'old' control cells (Fig. 4). However, the phosphoinositide metabolism was not as extensive as in fresh control cells; the final fraction of labelled PIP_2 was higher and that of labelled PA was lower in both ovalocytes and 'old' control cells compared to fresh control cells. This less extensive phosphoinositide turnover can most probably be attributed to the 'ageing' process of the cells during transportation, which reduced the energy level as well as the reduction potential of the cell.

4. Discussion

Similar to normal red cells, hereditary ovalocytic red cells change their shape upon metabolic depletion. However, in contrast to normal cells, the shape transformations are not very extensive; the ovalocytes retain their elongated stomatocytic character throughout depletion and only develop bulges on the membrane surface. This shape change behaviour indicates that ovalocytes go through shape transformations different from normal red cells and that it is unlikely that normal echinocytosis is balanced by the initial stomatocytic shape of the ovalocytes.

Based on the bilayer-couple hypothesis, it has been suggested that metabolically induced shape changes are correlated with the phosphoinositide metabolism and, in the case of echinocytosis, reduction of the area of the inner leaflet of the membrane [22,23]. Therefore it was surprising that the phosphoinositide metabolism of ovalocytes was very similar (if not identical) to that of normal cells. At the same time, this observation suggests that the phosphoinositide distribution between the two leaflets should be similar in these cells. If correct, it follows that the effect of degradation of PIP_2, PIP and PA to phosphatidylinositol and diacylglycerol, which should reduce the area of the inner leaflet to the same extent in ovalocytes as in normal cells, somehow is counteracted in the ovalocytes.

Hereditary ovalocytes are extremely rigid with a shear elastic modulus nearly four times that of normal red cells [7,8,12]. The rigidity is associated with the deletion of nine amino acids located between the N-terminal cytoplasmic and membrane domains, deleting a flexible hinge connecting these domains [35]. The structure of the membrane spanning domains of mutated band 3 is altered and the anion transport activity as well as the translational diffusion in the membrane are reduced [12,36,37]. In contrast, the N-terminal cytoplasmic domain appears not to be affected as mutated band 3 binds ankyrin with normal capacity and affinity [12,37].

Therefore it is likely that the abnormal mechanical properties of ovalocytic red cells are related to the increased stiffness of a normally flexible region of band 3. Exactly how is not known; it is possible that the translational constraints of mutated band 3 hinder normal movement of band 3-bound ankyrin required when the cell undergoes local deformation, as suggested by Schofield et al. [12]. Interestingly, mutated band 3 appears to have an increased propensity to aggregate and these oligomers are enriched in longitudinal strands of intramembrane particles [9,38], thereby preventing extension (and contraction) of the cytoskeleton and, thus, also cell deformation. In addition, the mutation may cause an entanglement of the band 3 with the cytoskeleton [11]. An impeded mobility of mutated band 3, whether through oligomer formation or interactions with the lipid bilayer or cytoskeleton, will obviously restrict the distortion that the cytoskeleton must undergo when the cell is deformed.

Upon metabolic depletion and concomitant degradation of the phosphoinositides, the area of the inner leaflet of the bilayer should shrink. However, the reduced area of the inner leaflet must be accompanied by a similar contraction of the cytoskeleton. Therefore it is possible that a cytoskeleton with a highly reduced ability to contract (or expand) would inhibit (or reduce) the loss of inner leaflet area. Consequently, such a cell would not be able to change its shape.

Since the turnover of the phosphoinositides in ovalocytes does not differ from that of normal red cells, it is evident that shape transformations of the red cell cannot be explained solely by the bilayer-couple hypothesis. Our results indicate that a fully flexible cytoskeleton is required for normal shape changes to occur, at least when induced metabolically.

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